YUVANESH RAJANTEREH B. ENG. (HONS) CHEMICAL ENGINEERING SEPTEMBER 2015

EXTRACTION OF ESSENTIAL OILS FROM *MELALEUCA ALTERNIFOLIA SP.* (AUSTRALIAN TEA TREE PLANT) USING SOLVENT EXTRACTION METHOD

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CHEMICAL ENGINEERING
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Dissertation submitted in partial fulfilment of
the requirements for the
Bachelor of Engineering (Hons)
(Chemical Engineering)

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CERTIFICATION OF APPROVAL

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Approved by,

____
(Prof. Dr. Thanabalan Murugesan)

UNIVERSITI TEKNOLOGI PETRONAS
BANDAR SERI ISKANDAR, PERAK
September 2015

CERTIFICATION OF ORIGINALITY

This is to certify that I am responsible for the work submitted in this project, that the original work is my own, except as specified in the references and acknowledgements, and that the original work contained herein have not been undertaken or done by unspecified sources or persons.

YUVANESH A/L RAJANTEREH

ABSTRACT

In this report, the *Melaleuca alternifolia* genus, commonly known as the Australian tea tree plant were chosen for essential oil extraction study, as the oil being extracted from this plant possess natural therapeutic medicinal values which serves as an anti-fungal, anti-microbial and anti-inflammatory agent to fight against various infections and illnesses. The major component in the tea tree oil (TTO) that largely contributes to its medicinal function is based on terpinen-4-ol components, which constitutes about 40.02% from the overall composition of the TTO. Other components, such as 1,8-cineole, also reveals antimicrobial properties, similar to those of the terpinen-4-ol components. In this research, the extraction of the tea tree oil, via solvent extraction method, is studied and the percentage yield of tea tree oil extracted is determined and the composition of tea tree absolutes (terpinen-4-ol, 1,8-cineole, γ-terpinene and α-terpineol) extracted was compared with standard tea tree oil as well as with ISO 4730 *Melaleuca* oil standard via GC-MS technique. A feasibility study was also conducted to compare the choices and favourability of different types of solvents (n-hexane, petroleum ether, ethanol and methanol) being studied in the solvent extraction method to identify the desired selectivity of solvents resulting in higher percentage yield of the tea tree oil, without any deterioration in the extracted oil quality. The selectivity of petroleum ether as compared to n-hexane, ethanol and methanol, was much higher, as the yield of tea tree absolute was profoundly higher (1.06 % on a wet basis) as compared to the rest of the solvents. The composition of terpinen-4-ol, 1,8-cineole and α-terpineol found in the tea tree extract based from the studied solvents were in the range of 17.22-39.34%, 0.83-1.46% and 2.17-3.70%, however the γ-terpinene compound were significantly low (1.07-3.28%) as compared to its composition in the standard tea tree oil (20.47%).

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CHAPTER 1
INTRODUCTION

1.1 Background of Study

In terms of its tropical biodiversity, Malaysia has a living heritage of its vast diversified plant species, in which amongst them consist of natural herbs, acquiring the medicinal and therapeutic properties as well as many other non-medicinal benefits. Historically, herbal plants tend to be an easily accessible and instantaneous type of medicine which were commonly being introduced for healthcare, as an ailment to prevent and treat various kind of illnesses. Apart from their therapeutic benefits, various parts of these herbal plants were also beneficial in terms of supply in dietary (food consumption), utilities for construction materials, ritual purposes, dyes and also for cosmetic purposes. Currently, in Malaysia, the demand for natural health supplements have been increasing over the years, which mainly shifts the interest on the exploration of herbal plants, to understand the medicinal properties of these herbs. For instance, some of the available herbaceous plants such as *Tongkat Ali (Eurycoma longifolia), Ginseng (Ginseng baloba), and turmeric (Curcuma domestica)* converted the common traditional uses into essential herbal product for healthcare purposes.

Melaleuca alternifolia sp. or commonly known as the Australian tea tree plant, is among one of the plant that distinguishes its therapeutic properties as the plants mentioned previously. Initially, this plant genus was originated from the land of Australia in New South Wales, which were widely used by the indigenous people there (Ismail, 2013). This plant genus belonged to the same family as the *eucalyptus* genus and was first discovered by Captain James Cook in the 1770's. The essential oil extracted mainly from the leaves of *Melaleuca alternifolia sp.* genus contains medicinal and therapeutic values which act as an antifungal, antibacterial and anti-inflammatory medication, commonly used to treat acne, athlete's foot, toenail fungus, yeast infections, cold sores and mouth ulcers. Besides that, this tea tree oil has also been used in the perfumery industry, in the control of wood-rot fungi, act as an antiseptic in water-gel fire blankets and also to treat *Legionella sp.* in air-conditioning systems (Johns, Johns, & Rudolph, 1992). According to (Carson, Hammer, & Riley, Compilation and Review of Published and Unpublished Tea Tree Oil Literature, 2005), unlike other medication oil, the tea tree oil extracted from the plant genus does not cause any irritant reactions when being applied to skin / dermal tissues as this is due to the lower concentration of 1,8-cineole composition, as stated in the ISO 4730 "Oil of *Melaleuca*-terpinen-4-ol type" (ISO/TC54, 2007).

The *Melaleuca alternifolia sp.* are described as a tall shrub (or small tree) up to a height of 7 m and with pointy narrow leaves which helps to differentiate from the other species of the same family plant, *Melaleuca linariifolia sp.,* which has much wider leaves and flattish-spherical fruits. Figure 1.1 below shows an example of the Australian tea tree plant (*Melaleuca alternifolia sp.)* which is being used as the sample raw material throughout the duration of this project.

FIGURE 1.1 Tea Tree Plant (Melaleuca alternifolia sp.)

1.2 Problem Statement

In Malaysia, the current available technology being upheld in the industry for the essential oil extraction from *Melaleuca* genus are by using steam distillation method, solvent extraction method, CO_2 supercritical fluid extraction and also the microwave technology (Schaneberg & Khan, 2002).

The most conventional method being applied by most of the industries related to this process till today is to extract essential oils by means of steam distillation technique. Based on the research study conducted by (Huynh, Phan, & Thieu, 2012), steam distillation technique is one of the oldest form of essential oil extraction, which requires simple utilities and offers the best route for distilling materials, consisting of raw plant materials. This method was established as a common practice of extraction by most of the industries as it only causes minimum changes to the oil composition as well as the medium used for extraction (the steam) is readily available, cheap, and can be reused throughout the process. According to (Carson, Hammer, & Riley, 2006), the current yield of *Melaleuca* essential oil is typically found to be about 1-2%, determined via this technique, as a basis of wet material weight.

As an alternative mode of extraction, other methods such as solvent extraction method are also considered, in order to evaluate the effectiveness of essential oil extraction, as compared to the conventional technology. This mode of extraction is advantageous for the production of essential oil in the perfumery industries and supposedly to deliver oil almost closely matching to the in-situ leaf oil composition (Baker, Lowe, & Southwell, 2000). However, in the current approach, this mode of extraction is only being carried out on lab-scale purposes, due to the chances and uncertainty in which the oil composition extracted via this method might conflict with the standard composition of tea tree oil as mentioned in the ISO 4730 "Oil of *Melaleuca*-terpinen-4-ol type" (Ginn, 2009).

Therefore, one of the challenges in this extraction process is to find an alternative extraction method which could be applied on a larger industrial scale, which may probably contribute to a higher yield of therapeutic tea tree oil as compared to the traditional steam distillation method. The real challenge of proposing a different method of extraction is that the extracted tea tree oil composition via the proposed technique should at least satisfy the standard tea tree oil composition as stated in the ISO 4730. Hence, in this project, the solvent extraction technique is chosen to be studied, in terms of determining the yield of essential oil being extracted from the *Melaleuca* plant as well as the identification of the major components in the extracted oil, based on different types of solvents, including both polar and non-polar solvents.

1.3 Objectives

The main objectives of this report are:

- To determine the yield of essential oil extracted from tea tree plant sample via different types of solvents (both polar and non-polar) on a basis of wet material weight

- To identify the major and minor chemical constituents (terpinen-4-ol, 1,8-cineole, γ-terpinene & α-terpineol) in the extracted tea tree oil using Gas Chromatography-Mass Spectrometer (GCMS) method

- To compare the chemical composition of the extracted tea tree oil with the standard composition of tea tree oil as regulated in ISO 4730 “Oil of Melaleuca-terpinen-4-ol type”

1.4 Scope of Study

The scope of study for this project is restricted to only understand and analyse the selected major and minor components in *Melaleuca* oil / tea tree oil extracted using solvent extraction, particularly via Soxhlet technique, which are primarily terpinen-4-ol, 1,8-cineole, γ-terpinene and α-terpineol, which are then compared with the standard tea tree oil composition as stated in ISO 4730. The yield of essential oil extraction are then determined and compared based on different types of solvents, using both polar (ethanol and methanol) and non-polar (n-hexane and petroleum ether) solvents.

1.5 Relevance and Feasibility

As compared to solvent extraction method, the traditional distillation technique utilizes high temperature steam as the extraction medium to force the tiny intercellular pockets that trap the essential oils to open and thus releases them. The temperature of the steam should be sufficiently high, high enough to exert adequate force onto the intercellular pores to provide an opening for the essential oils to diffuse out. This may create a tendency for the heat of steam to cause changes in the overall composition of the extracted oil, although at a minimum level, which it may affect the overall functionality or properties of the extracted therapeutic oil. Besides that, using water alone as a solubilizing medium for the overall extraction process, is not adequate enough as mostly, the steam only manage to capture the polar and semi-polar constituents, but not the lipophilic compounds such as terpenes, which are almost insoluble in water but solubilize well with most organic solvents (Saller, Berger, Reichling, & Harkenthal, 1998).

Alternatively, the application of the proposed extraction method is highly relevant and feasible for some plants (including tea tree plant) that have much smaller oil sacs which makes the extraction process to be slightly difficult, as well as, they can be considered to be fragile and delicate which contributes to the tendency for their fragrances to be easily damaged due to the extreme heat using the conventional method. Therefore, it provides the relevancy for using the latter method to extract the medicinal essential oil which naturally present in small quantities in tea tree plant. As compared to steam distillation method, solvent extraction is adequately feasible for the tea tree oil extraction due to shorter duration of the extraction process and more cost-efficient in terms of their operating cost in which in steam distillation method, the power consumption is much higher to boil water, instead of power consumed to boil low-boiling point organic solvents.

CHAPTER 2

LITERATURE REVIEW

2.1 Essential Oils

Essential oils (essences = volatile oils) represents the active constituents / chemical compositions of herbaceous plants which are mainly secreted in their leaves, roots, stems, flowers, schizogenous or lysogenous glands (Telange & Pethe, 2013). The term "essential oil" was first coined in the 16th century by a Swiss reformer of medicine, Paracelcus von Hohenheim, as he claimed the effective components of a drug to be *Quinta essentia* (Guenther, 1950). According to the Standards Association of Australia in 1968, the term essential oils are defined as "volatile oils, in which most of them are naturally odorous, which are found in certain plants or specified parts of plants and extracted based on acceptable procedures, in such a way that the nature and compositions of the extract obtained remains unchanged". The proposed definition is an utmost significant statement as the composition of essential oils being extracted contains the DNA of the herbal plant itself.

As claimed by (Burt, 2004), the conventional steam distillation method is the most common practice being exerted to produce essential oils on a commercial basis. Nevertheless, a significant difference in terms of the composition of oils retrieved by solvent extraction as compared to steam distillation is concluded, which may relatively impact the antimicrobial properties of the extracted oil. This theory is further reinstated by (Packiyasothy, E.V., & Kyle, 2002) in which herbal essential oils extracted via solvent extraction (using hexane) had shown to present greater antimicrobial activity than the extracts derived from steam distillation.

2.2 Australian Tea Tree Plant (*Melaleuca alternifolia sp.*)

The Australian tea tree plant (*Melaleuca alternifolia sp.)*, from the *Myrtaceae* family, an aromatic and herbaceous plant genus, which is best known for the production of herbal essential oils due to its medicinal and therapeutic constituents in the plant compositions. Apart from its medicinal value, the remaining parts from this plant are also beneficial for various applications, for instance, the branches from this plant are utilized for the broom fence production, bark paintings, sealing and insulation from their many coloured barks, fuel and construction materials from their wood as well as the honey extract from the nectar (Boland, et al., 2006). Initially, the existence of this plant was first discovered in the subtropical costal region of New South Wales in Australia by Captain James Cook during his exploratory voyage in 1770, when he came across a myrtaceous shrub (probably a *Leptospermum,* under the *Melaleuc*a genus) with leaves that were consumed by his sailors as a substitute for tea. The plantings of this herbaceous species were then later extended to the United States, Zimbabwe, New Zealand, China, India and other parts of the nation. A full grown *Melaleuca alternifolia sp.* is established to be a height of within 3 to 8 meters with a shrub-like growth as shown in Figure 2.1.

FIGURE 2.2 *Melaleuca alternifolia* shrub-like growth

2.3 History of Melaleuca alternifolia sp. usage

The healing properties of the *Melaleuca* leaves were first revealed by the indigenous Australian Bundjabung Aborigines since several thousand years ago in the north of New South Wales. The natives exploit the leaves from the plant as a traditional remedy for many medication purposes, for instance, munching the young leaves to relieve headaches, used as an ailment for common colds, sore throat, insect bites, wounds or fungal skin infections as well as in delousing (Clarke, 2011). (Mackenzie, 2006) had also claimed that the native people apprehend the herbal extract from the tea leaves by crushing them to be used as rubbing mediums, mixed the leaves with clay to form poultices and also utilizes the water that was collected under the tea trees for bathing purposes. Once the members of Captain James Cook set foot on the land of Australia in 1770s, they observed the application of these tea tree leaves by the indigenous people and learned to use those leaves for their own ailment application, once understanding the medicinal value of the herbs.

Subsequently, in 1922, Arthur Penfold, an Australian chemist, and his team, from the Sydney Museum of Technology and Applied Science, successfully managed to extract the oil from *Melaleuca alternifolia sp.* and published a research paper which claimed that the extracted oil is able to act as both antimicrobial and antifungal mechanism for a wide range of infections (Mackenzie, 2006) (Saller, Berger, Reichling, & Harkenthal, 1998). After the successful publication by Arthur Penfold, the Australian Tea Tree Oil were then recognized around the global for its magnificent therapeutic and medicinal properties for treating all sorts of infections and infestations including from cuts and wounds to head lice, ringworms, leg ulcers, catarrh, thrush, tonsillitis, pyorrhoea and gingivitis. However, during the period of 1939 – 1945, the Second World War, the production of *Melaleuca* oil was in limited supply as all the available stocks were utilized to prevent the spread of the infections from the unavoidable war wounds, both from the soldiers and munitions workers' that were assisting during the war effort.

The medicinal properties of these *Melaleuca alternifolia sp.* were then being further investigated in the early 1990s, by a scientific research team under the lead of Associate Professor Tom Riley from University of Western Australia. In their past research experiences, they continue to popularize the versatility and efficiency of this traditional herbal oil by highlighting the oil's healing tendency to fight against infectious diseases and inflammatory circumstances. Sir Tom Riley and his team was the first group of people to found the cure for methicillin-resistant Staphylococcus aureus, also known as the hospital superbug (MRSA), as the tea tree oil showed a remarkable susceptibility in suppressing the growth of the infection. This finding were first brought to their attention during their research investigation in understanding the antimicrobial activity of the major components in the medicinal oil of *Melaleuca alternifolia sp.*

According to (Carson, Cookson, Farrelly, & Riley, 1995), staphylococcus strain of bacterium managed to suppress the antimicrobial activity in most of the synthesized antibacterial medicines available from the orthodox medicine. This type of infection commonly infiltrates in those suffering from skin lesions, especially from post-operative wounds and / or a depressed immune system, and it could be transmitted from patient to patient via hospital staff and on equipment such as pens and stethoscopes (MRSA carriage). However, with the detailed research study developed by Sir Tom Riley and his team, the MRSA infections could be suppressed with the successful treatment of *Melaleuca* essential oil's antimicrobial components on the MRSA carriages. Hence, till today, various method of distilling the essential oil extract from the *Melaleuca alternifolia* leaves are being carried out across the global due to its significant role as a natural herbal remedy with tremendous healing powers.

2.4 Chemical Constituents of *Melaleuca* oil / Tea tree oil

The tea tree oil extracted from the *Melaleuca* leaves consist of a complete mixture blend of approximately 100 different types of compound in its compositions. It is composed of terpene hydrocarbons, which are mainly classified as monoterpenes ($C_{10}H_{16}$), sesquiterpenes ($C_{15}H_{24}$) and their related alcohols (Carson, Hammer, & Riley, 2006). (Sharp, 1983) claimed that terpenes are known to be volatile and aromatic hydrocarbons and could be regard as polymers of isoprene, which has a molecular formula of C_5H_8. Among the different types of monoterpenes present in the composition matrix of the tea tree leaves are predominantly comprised of terpinen-4-o1, γ-terpinene, α-terpinene, 1,8-cineole, terpinolene, ρ-cymene, α-pinene, α-terpineol, aromadendrene, δ-cadinene, limonene, sabiene, globulol and viridiflorol. Figure 2.2 and Figure 2.3 describes the chemical structures of the main constituents present in tea tree oil.

However, the antimicrobial / antifungal properties of this natural herbal plant is commonly influenced by the significant compositions of terpinen-4-ol, 1,8-cineole and also based on the inter-linking relationship of the other mentioned components as well. Despite of their significance in governing the efficacy of their antimicrobial properties, the composition of these major components (terpinen-4-ol and 1,8-cineole), as well as the other components, are regulated under the International Standard of Organization (ISO 4730) and the Australian Standard AS 2782-2009, set forth as a benchmark study for standard TTO composition, which is known as "Oil of *Melaleuca* –terpinen-4-ol type". This standard provides a range of maximum and/or minimum compositions for 14 components of the TTO as depicted in Table 2.1 (ISO/TC54, 2007).

TABLE 2.1 Composition of M. alternifolia (tea tree) oil

Component	Composition (%)	
	ISO 4730 range	Typical Composition
Terpinen-4-ol	≥ 30	40.1
γ-Terpinene	10-28	23.0
α-Terpinene	5-13	10.4
1,8-Cineole	≤ 15	5.1
Terpinolene	1.5-5.0	3.1
ρ-Cymene	0.5-12	2.9
α-Pinene	1.0-6.0	2.6
α-Terpineol	1.5-8.0	2.4
Aromadendrene	Trace-7	1.5
δ-Cadinene	Trace-8	1.3
Limonene	0.5-4.0	1.0
Sabiene	Trace-3.5	0.2
Globulol	Trace-3	0.2
Viridiflorol	Trace-1.5	0.1

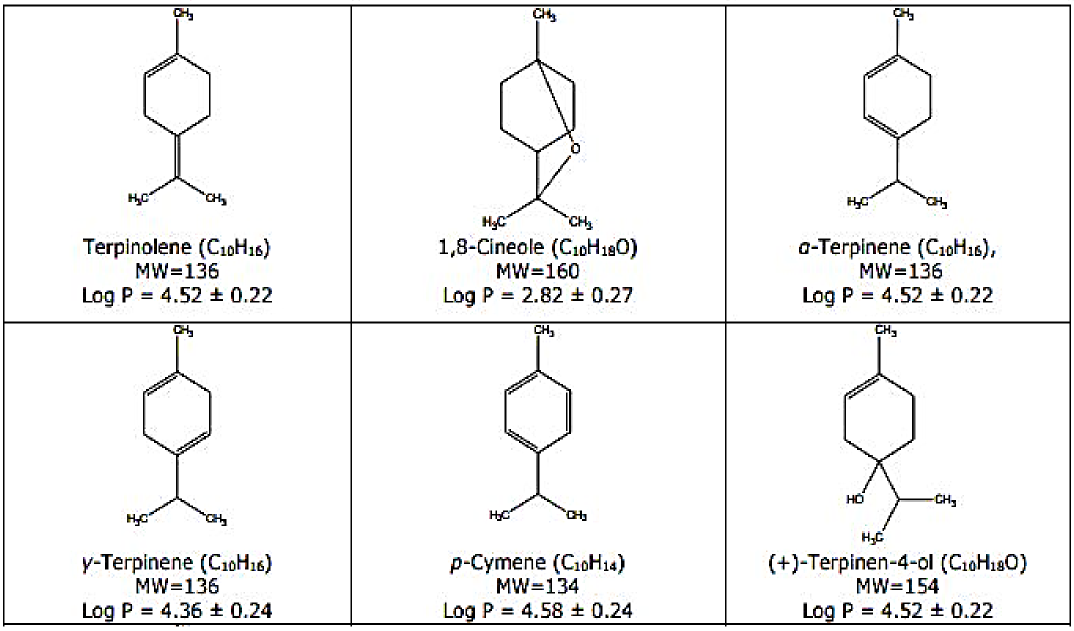

FIGURE 2.3 Chemical structures of the major constituents in tea tree oil

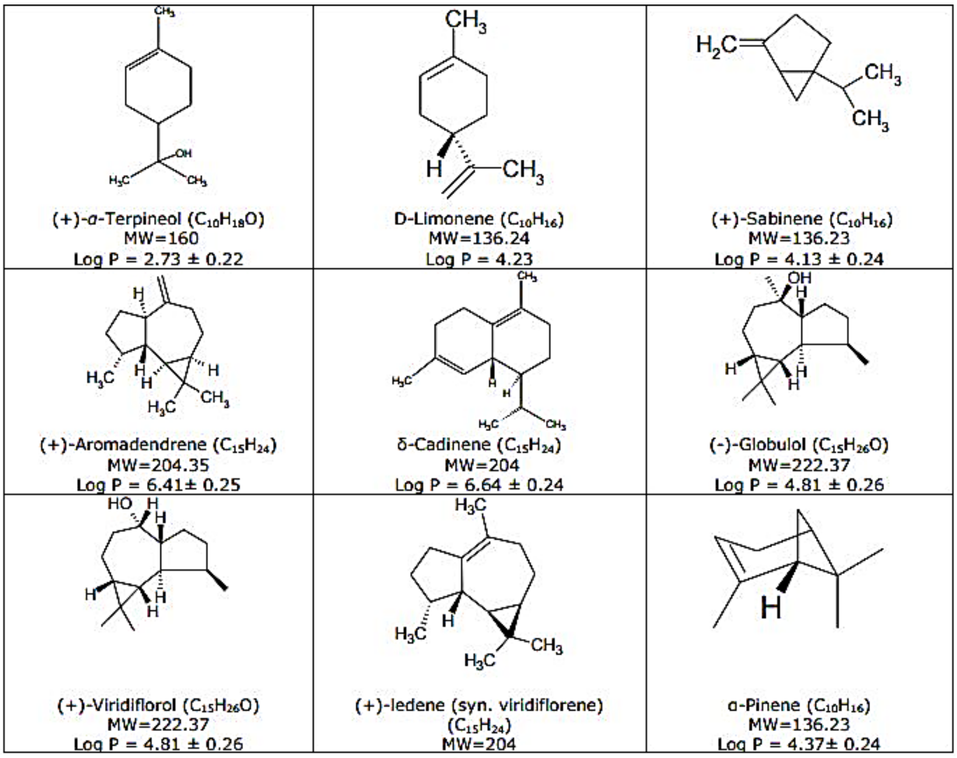

FIGURE 2.4 Chemical structures of major constituents in tea tree oil

Based on (Saller, Berger, Reichling, & Harkenthal, 1998), the standardized value shown above provides constraints, such that, the minimum composition of terpinen-4-ol should be at least 30% while the maximum composition of 1,8-cineole should not be more than 15%. The latter component was recommended at a lower percentage as 1,8-cineole could be a source of irritant when exposed to skin surface at a higher concentration. This standard is vital and necessary as it provides an overall concept on the physical and chemical standards for the desired chemotypes. According to (Carson, Hammer, & Riley, Compilation and Review of Published and Unpublished Tea Tree Oil Literature, 2005), there are several types of chemotypes of *M. alternifolia,* with each producing oil with a different chemical composition. Till present research, six chemotypes have been identified, which are, terpinen-4-ol chemotype, terpinolene chemotype and four 1,8-cineole chemotypes.

Despite of the relative compositions of different types of components in this *Melaleuca* species, there is still some uncertainty in justifying a concrete evident on identifying the corresponding components in TTO which are correlated to the antimicrobial properties of the essential oil. The complexity of the mixtures of compositions in TTO signifies that certain combinations of components are more likely to be prone in contributing to the antimicrobial properties of the tea tree oil. For example, (Carson, Hammer, & Riley, 2005) was ascertained that terpinen-4-ol and α-terpineol claimed to be the dominant factors in resulting to the antifungal and antibacterial action. Besides this combination, it is also posed that α-pinene component are also able to encounter the microbial activities, as well as, with the combination of linalool and limonene components (Sikkema, de Bont, & Poolman, 1995). Hence, apart from focusing on the antimicrobial property of the main ingredient in tea tree oil (terpinen-4-ol and 1,8-cineole), the synergistic relationship between other related compositions should also be accounted to possibly yield the medicinal properties from the natural oil extract.

2.5 Available Extraction Methods for Tea Tree Oil

Previously, the common practice being used for the extraction of tea tree oil from *M. alternifolia* plant is by using the steam distillation method. This conventional method is one of the ancient ways of essential oil extraction, as the concept of extraction is claimed to be direct, least complex and considered as one of the best method to distil raw materials from plants (especially leaves). Nevertheless, with the advancement of current research and technology, other methods are also being considered in this process such as solvent extraction method, CO_2 supercritical extraction, microwave technology, and also enfleurage method. However, for this project purposes, only solvent extraction method is being emphasized for the extraction study and will be explained in-depth in the following section.

2.5.1 Solvent Extraction Method

Despite of this current modern essential oil extraction in the perfumery industry as claimed by (John, 2003), the existence of this solvent extraction technique had already been discovered in 1835. The necessity of this technique emerged as some plant materials undergo degradation due to the high heat carried by the heated steam in the steam distillation technique, leading to substantial loss in the essential oil recovery (Benedict, 2009). Hence, it is proposed that volatile solvents such as petroleum ether, n-hexane, ethanol or others could be utilized as a mode of oil extraction from these plant materials.

Based on (P.Skaria, 2007) (Benedict, 2009), using extraction of essential oils from flower materials, the fresh flowers are initially collected and charged into a specific extractor (Soxhlet chamber) at room temperature condition and are cautiously rinsed with purified hydrocarbon solvents. The plant material, in this case, the *Melaleuca alternifolia* leaves and small twigs, containing the desired essential compounds is placed into a thimble, which is constructed from thick filter paper and charged into the Soxhlet extraction unit. The Soxhlet unit is equipped with a top condenser and then being placed onto the opening of a round-flat bottomed flask containing the selected extraction solvent for the process, such as petroleum ether, n-hexane, ethanol, diethyl ether (B.Jensen, 2009). The choice of solvents to be used should encompass the following criteria / properties (Pengelley, 1996):

1. The solvent should completely dissolve all the required essential compounds from the plant within a short time, with a certain exception of other matters such as plant wax, pigments and albuminous compounds.
2. The solvent to be used should have sufficiently low and uniform boiling point, as it would be convenient to easily remove the solvent without subjecting to higher temperatures, which may cause losses in the recovered products.
3. The selected solvent should be hydrophobic, else the water content from the plant material may dissolve into the solvent, causing towards separation difficulties.
4. The selected solvent should also be chemically inert which is significant, in order to ensure there is no reaction and changes of the plant chemical constituents.

The extraction solvent is heated to reflux either by using uniform heating through water bath or insulated electric heater to maintain uniform heat transfer to the solvent and also to minimize the heat loss to the surrounding. As the solvent is heated, the vapour phase travels up to the distillation arm and occupies the extraction chamber containing the raw materials of *Melaleuca* plant species. The condenser attached at the top of the extractor condenses the vapour and allows the condensed liquid to flow back into the chamber. As the condensation process is prolonged, the chamber containing the raw materials is continuously filled with the condensed warm solvent. As the tea tree plant is brought into contact with the warm solvent, the desired essential oil/compounds together with other components, will then dissolve into the warm solvent (B.Jensen, 2007). As the condensed warm solvent almost completely occupies the Soxhlet chamber, the solvent in the chamber is automatically emptied using the siphon side arm, which returns back the mixture of solvent and the extracted compounds into the boiling flask. This process is then repeated for many cycles which could take up to several hours or days. Figure 2.4 below shows an example of the Soxhlet extractor model setup, viable for the tea tree essential oil extraction.

For each cycle of extraction, a fraction of the non-volatile compounds diffuses into the recycled solvent. After several times of the recycling process, the plant extract, similarly known as concrete (a combination of wax, essential oils, and other dissolved organic matters) is claimed to be highly concentrated in the boiling flask. This mode of extraction is more advantageous as a fact that minimization in the amount of solvent used for this process is possibly achievable. This minimization is attainable as the solvent used for the extraction process is introduced once and continuously recycled several times, instead of introducing more portions of warm solvent to recover the plant extracts.

Once the concentrated product is collected in the boiling flask, the recycled solvent is removed by using a rotary evaporator under vacuum operation condition. This separation system is important and serves as an indicator to maintain the purity of the tea tree extract, for some of the raffinate (dissolved solvent with traces of solubilized organic matter) might be left with the concrete residue, which may jeopardize the quality and the functionality of the concrete residue as well as to maximize the yield of the concrete. Once the solvent are completely removed, the obtained concrete is dissolved using methanol, at a temperature of 40°C for 1 hour and subsequent refrigeration at -10°C to partially precipitate the wax (or other heavy residue molecules) from the concrete composition matrix (Rout, Naik, & Rao, 2008).

The mixture is then finally filtered through a chilled funnel and the methanol solvent is removed using the rotary evaporator, which results in the final desired product, the *Melaleuca alternifolia* essential oil compounds, together with some trace amount of waxes, which is referred to as "absolutes". The obtained product is then subjected for the compositional analysis using GC-MS method, in order to identify the major components, as mentioned before, as well as its corresponding proportion in the obtained compound.

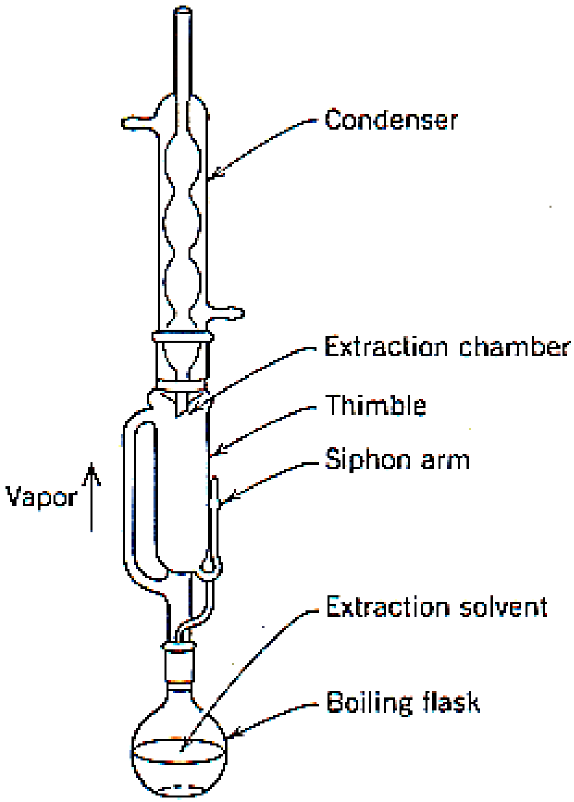

FIGURE 2.5 Solvent Extraction Setup

2.6 Characterization and Identification of Volatile Oil Contents

Identification of volatile oil contents can be performed with the utilization of gas chromatography-mass spectrometer technique as the development of this method has concluded considerable enhancement in the analysis of the chemical constituents of essential oils. In the current approach, Gas Chromatography-Mass Spectrometer (GC-MS) method is the most powerful analysis tool which utilizes the combination of effectively separating the different constituents of the sample and subsequent identification by mass spectrometry. Hence, in this research, Gas Chromatography-Mass Spectrometer technique is applied, as it is applicable in analysing the extracted tea tree oil due to its simplicity, rapidity and efficiency, as well as for both qualitative and quantitative analysis purposes of essential oil constituents and composition variations (David, Scanlan, Sandra, & Szelewski, 2002) (Chamorro, Zambon, Morales, Sequeira, & Velasco, n.d). As a sample constituents elutes from the GC column, it enters the ionization chamber of the mass spectrometer, in which the molecules are ionized, sorted each by their mass-to-charge ratio and measures their molecule weights (Rajantereh, 2012).

FIGURE 2.6 Modern GC-MS Instrument

CHAPTER 3
METHODOLOGY

3.1 Preparation of sample

The tea tree plant sample (*Melaleuca alternifolia sp.*) were obtained from the Tambun Acupuncture Academy, Perak, which is the raw material to be studied in this research work. The obtained tea tree plant were collected and cleaned by washing in water before subjecting for oven-drying at 40^oC (Lemos, Melo, Rocha, Barbosa, & Pinheiro, 2012). Figure 3.1 shows the collected tea tree plant from the academy prior to washing and oven-dried at the stated temperature. The plant samples are kept in the oven until a constant weight is achieved.

The plant were then cut into smaller stem twiglets and stored into an air-tight container. The main woody part of the plant was not considered during the sample preparation process because there is no oil content along that region, which is reinforced by (Southwell & Lowe, 1999). The dried tea tree plant were then granulated into tea tree powder by using a grinder. The powdered sample were then further refined by manual sieving using a 1-mm size sieve metal plate. The sieved tea tree powder were then kept in the oven at the same temperature (40^oC) until a constant weight is achieved before being subjected for the solvent extraction process. Figure 3.2 shows the sieved 1-mm size of tea tree powder prior subjecting to the solvent extraction process.

FIGURE 3.1 Fresh tea tree plant (Melaleuca alternifolia sp) collected from Tambun Acupuncture Academy

FIGURE 3.2 1-mm size tea tree powder after manual sieving

3.2 Solvent Extraction Method

As mentioned in Chapter 2 previously, the type of solvent extraction method chosen for this research work is by using the Soxhlet extraction technique. The following illustration (Figure 3.3) demonstrates the setup of Soxhlet extractor model for this research work using different types of extraction solvents, both polar and non-polar (n-hexane, petroleum ether, ethanol and methanol), to extract the essential oil of *Melaleuca alternifolia* (tea tree plant).

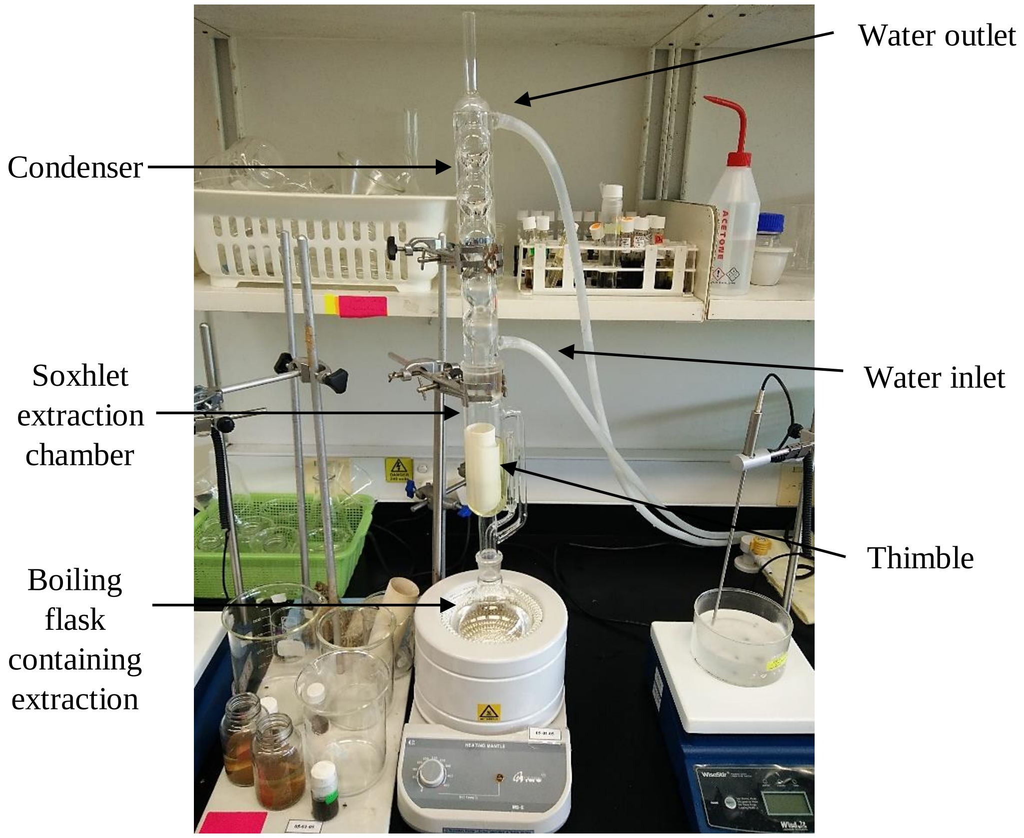

FIGURE 3.3 Solvent Extraction Lab Setup

3.3 Experimental Procedures

1. 50 g of tea tree powder is measured and weighed and subsequently transferred into the extraction thimble. The extraction thimble, containing the sample, is then placed into the Soxhlet extraction chamber.
2. A 500 mL round-bottomed flask is used as the boiling flask and as a reservoir for the extraction solvent. 300 mL of n-hexane solvent is then measured and transferred into the flask.
3. The solvent is heated at its boiling point (n-hexane = 69.7°C) and the extraction system was left to run under reflux condition, for a total of 6 reflux cycles.
4. Once the extraction is completed, 2.5 mL of the tea tree extract is taken for the GC-MS analysis and the remaining extract were stored in a container.
5. The experiment were then repeated using the same sample size with petroleum ether, ethanol and methanol (Prakash, Rout, Chanotiya, & Misra, 2012).

Figure 3.4 below shows the obtained tea tree extract after the plant sample were extracted using n-hexane solvent under the Soxhlet extraction technique.

FIGURE 3.4 Tea tree extract spent with n-hexane

3.3.1 Separation Process for Solvent Extraction

Once the tea tree extract (containing the essential oils, waxes, resin compounds as well as spent solvent) were collected from the Soxhlet method, the combined extract were subjected to solvent removal using a rotary evaporator under vacuum condition at 40^{o}C, which is essential in order to lower down the boiling point of solvent. Figure 3.5 below shows the rotary evaporator used as the separation system of n-hexane solvent from the extracted tea tree extract; Figure 3.6 portrays the recovery of n-hexane solvent and Figure 3.7 describes the appearance of tea tree concrete (combination of tea tree oil, waxes, albuminous and resin compounds) after the separation process.

FIGURE 3.5 Rotary evaporation for n-hexane removal from tea tree extract

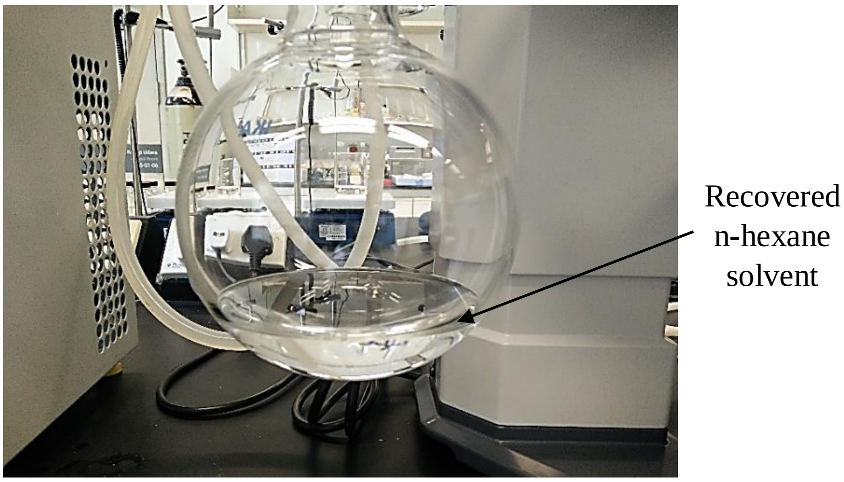

FIGURE 3.6 Recovered n-hexane solvent from tea tree extract

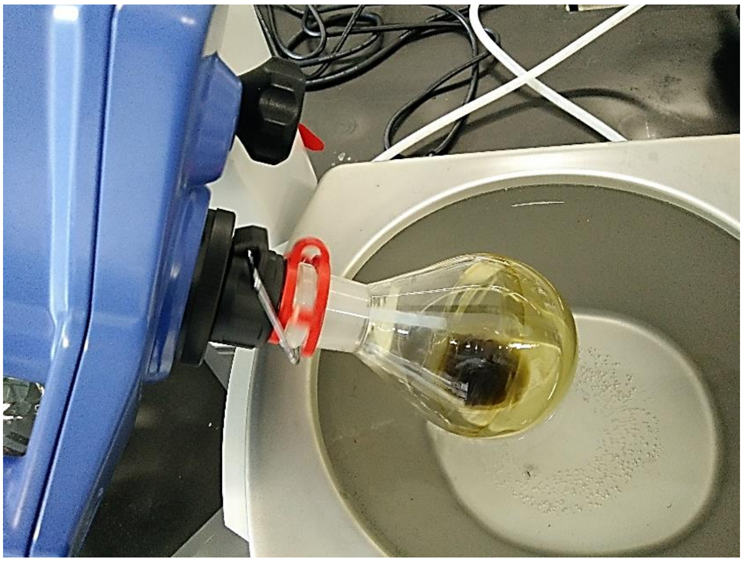

FIGURE 3.7 Tea tree concrete residue after n-hexane removal

Once the tea tree concrete residue is obtained after the solvent removal process, the concrete oil was dissolved in minimum volume of absolute alcohol to remove the natural waxes present in the essential oil, which in this case, 6 mL of methanol solvent is used for dissolving the collected essential oil (Khan & Rehman, 2005). The dissolved solution is then gently heated at 40°C for 30 minutes, followed by subsequent refrigeration at -15°C for 24 hours, which is significant to partially precipitate the waxes and other resin molecules. The following figure (Figure 3.8) illustrates separation / precipitation of waxes and other heavy molecules once the mixture is cooled for 24 hours.

FIGURE 3.8 Precipitated waxes after mixture is cooled for 1 day

The mixture is then further subjected for vacuum filtration, to separate the essential oil containing methanol from the waxes and other heavy molecules. Figure 3.9 demonstrates the vacuum filtration setup for the separation of essential oil from the waxes as well as other heavy molecules. Once the filtration process is carried out, the obtained solution is then again subjected for rotary evaporation to remove the methanol solvent, which finally results in the produced essential oils together with some traces of wax, which is termed as “absolutes”. Figure 3.11 shows the recovery of absolutes after performing the rotary evaporation to eliminate the methanol solvent. The recovered absolute is then diluted with extraction solvent (n-hexane, petroleum ether and ethanol), in which, 2.5 mL sample is taken for the characterization study using GC-MS to identify the major components in the extracted absolute as well as their corresponding compositions.

FIGURE 3.9 Vacuum filtration setup to separate waxes from essential oil

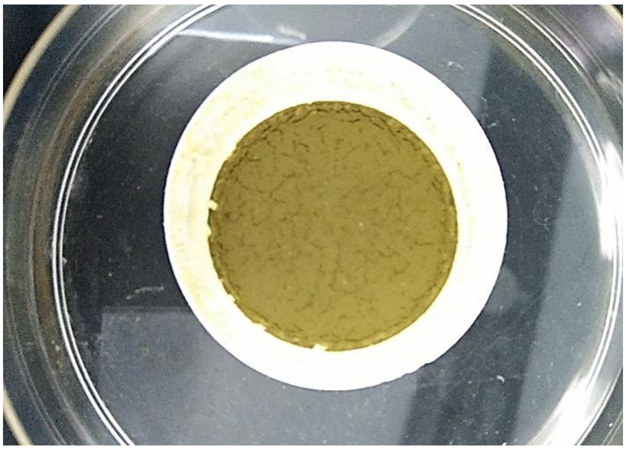

FIGURE 3.10 Filtrated waxes after vacuum filtration

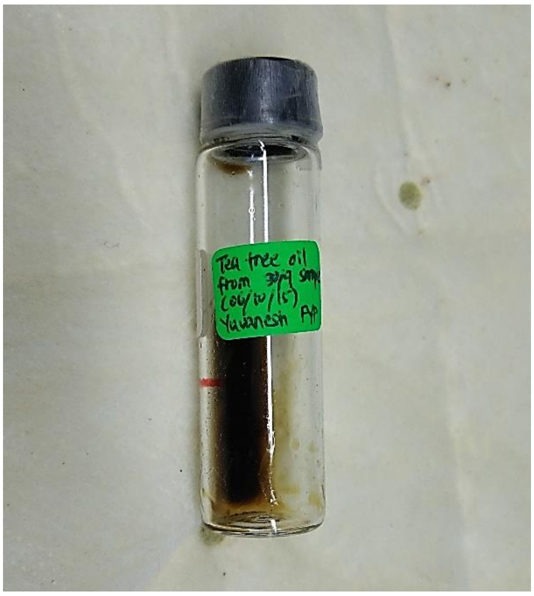

FIGURE 3.11 Recovery of absolutes after methanol removal

3.4 Characterization of the essential tea tree oil using GC – MS method

The commercial tea tree oil sample was purchased from Biossentials Sdn. Bhd. Malaysia, which was used as a reference standard to compare with the yield produced from solvent extraction. The standard tea tree oil was diluted with the extraction solvent (n-hexane, petroleum ether, ethanol and methanol) using a ratio of 1:10 (v/v) prior subjecting to Gas Chromatography-Mass Spectrometer analysis (Tranchida, et al., 2010). 2.5 mL of the tea tree extract and 2.5 mL of diluted tea tree oil was subjected to the GC-MS characterization study and comparison was made based on the composition peak given by the standard solution.

The Gas Chromatography-Mass Spectrometer analysis was performed using AGILENT 7890B GC instrument equipped with an AGILENT 5975C mass-specific detector and an AGILENT 7693 auto-sampler, under the following operating parameters:

Column	: AGILENT J&W HP-1 fused silica capillary column (60 m x 0.25 mm i.d.) coated with a 0.25 µm film of cross-linked 100% dimethyl-polysiloxane
Carrier gas	: Helium (constant pressure of 24 psi)
Column temperature	: 50°C (for 2 min), to 180°C (2.0°C / min)
Injector temperature	: 250°C
Split ratio	: 25:1

For the scan measurements, the spectra were recorded at 70 eV from m/z 35 to 450. The compound identification is done based on the comparison of the spectra with the databases (Wiley and NIST). The unknown peaks were further identified based on the relative retention indices compared with literature and the reference standard of tea tree oil purchased from Biossentials Sdn Bhd (Wang, et al., 2015).

3.5 Project Activities and Key Milestones

The main path of this project is to determine the yield of essential oil extraction from the *Melaleuca alternifolia* plant by using solvent / soxhlet extraction method. Prior to the extraction method, the research work deals in attaining and preparation of the required plant sample, which is necessary for the essential oil extraction purposes. The obtained yield is then further subjected for the characterization study, which deals with the compositional analysis using Gas Chromatography Mass Spectrometer, to identify the possible major components in the essential oil extract which may constitute to its antimicrobial properties. Once the identified components, as well as their corresponding compositions, are determined, a comparison study is done based on the standard composition of the tea tree oil. The key milestones of the research project is as shown in following Table 3.1.

3.6 Project Gantt chart

Table 3.1 describes the overall Gantt chart being constructed for this research project.

TABLE 3.1 Project Gantt chart

FYP I

No	Project Activity	FINAL YEAR PROJECT I													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Introductory Lecture with the FYP Coordinator	■													
2	Title Selection		■												
3	Confirmation of Project Topic			■											
4	First meeting with Supervisor			■											
5	Preliminary Research Work				■	■	■	■	■	■	■	■	■	■	■
6	Submission of Extended Proposal							■							
7	Proposal Defence									■					
8	Project Work Starts										■	■	■	■	■
9	Submission of Interim Draft Report to Supervisor												■		
10	Submission of Final Interim Report (After Correction)													■	
11	Submission of Marks by Supervisor														■

FYP II

No	Project Activity	FINAL YEAR PROJECT II													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Collection of plant sample from Tambun Acupuncture Academy	■	■	■	■										
2	Solvent Extraction using n-hexane, petroleum ether and ethanol solvents		■	■	■	■	■								
3	Characterization study on extracted oil using GC-MS						■	■							
4	Submission of Progress Report								●						
5	Solvent Extraction using methanol, n-butane and n-pentane solvents									■	■				
6	Characterization study on extracted oil using GC-MS									■	■				
7	Pre-SEDEX											●			
8	Submission of dissertation												●		
9	Submission of technical paper												●		
10	Viva													●	
11	Submission of hardbound dissertation														●

● Key Milestones

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Results and Discussions

The chromatographic profile below (Figure 4.1, Figure 4.2, Figure 4.3, Figure 4.4 and Figure 4.5) reveals the components of interest present in the extracted tea tree absolutes based on the different types of solvents used as well as their corresponding compositions. For an overall discussion purpose, the compositions of the components of interest from the standard tea tree oil and the tea tree absolutes extracted were obtained and tabulated in Table 4.2 and the results were further discussed in the following section.

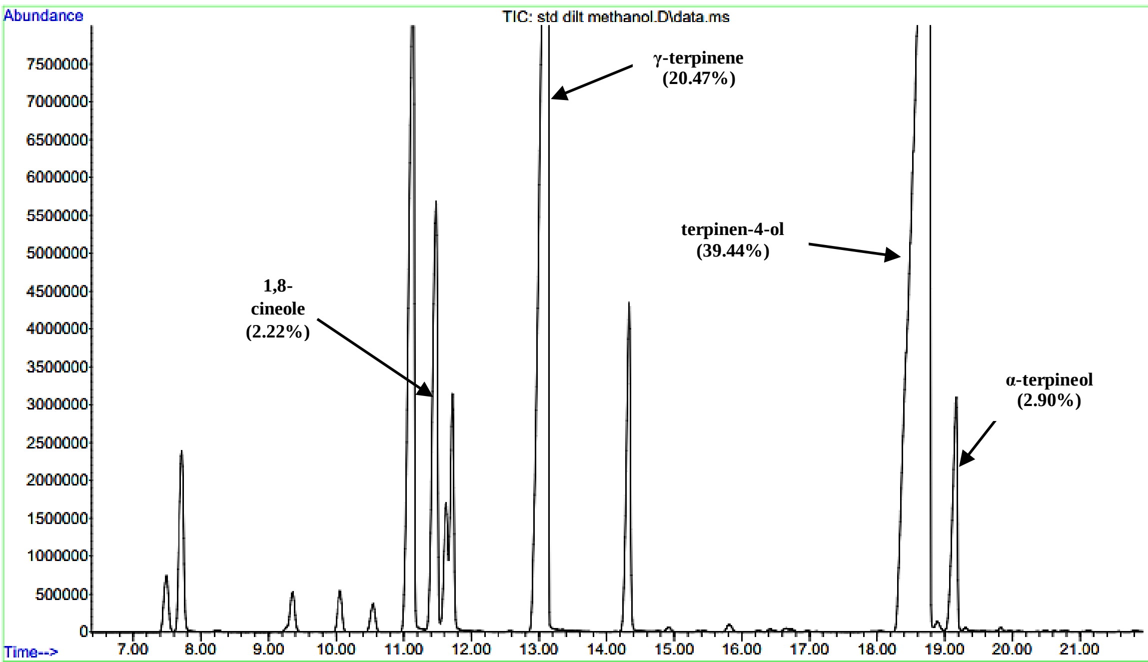

FIGURE 4.1 Chromatographic profile of standard tea tree oil

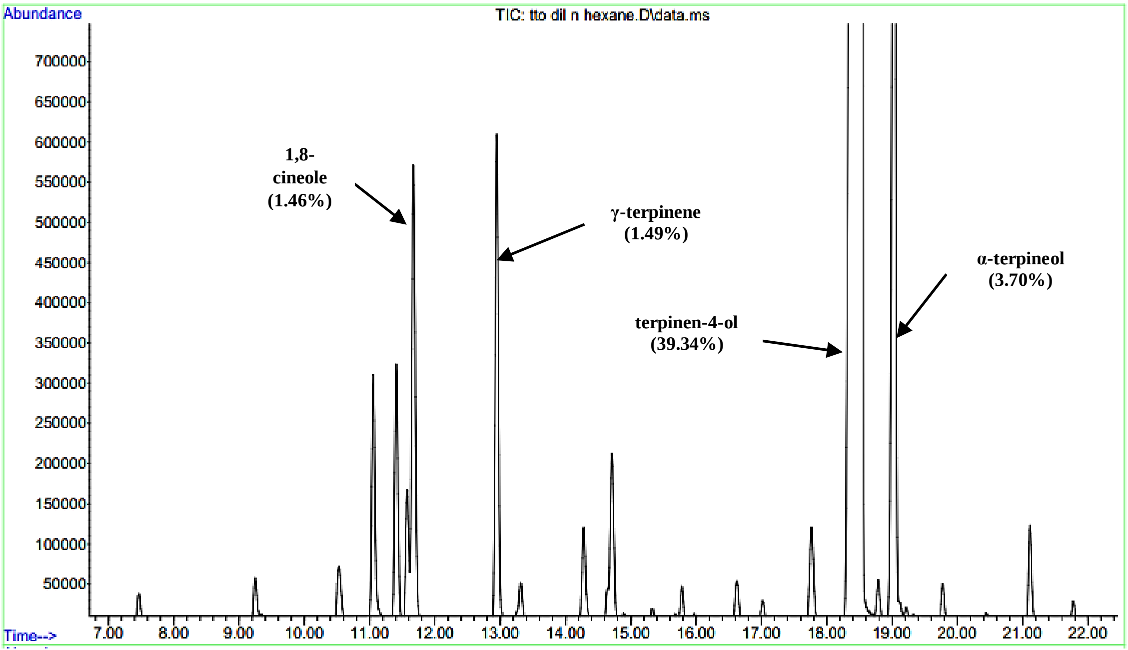

FIGURE 4.2 Chromatographic profile of tea tree absolutes from n-hexane extraction

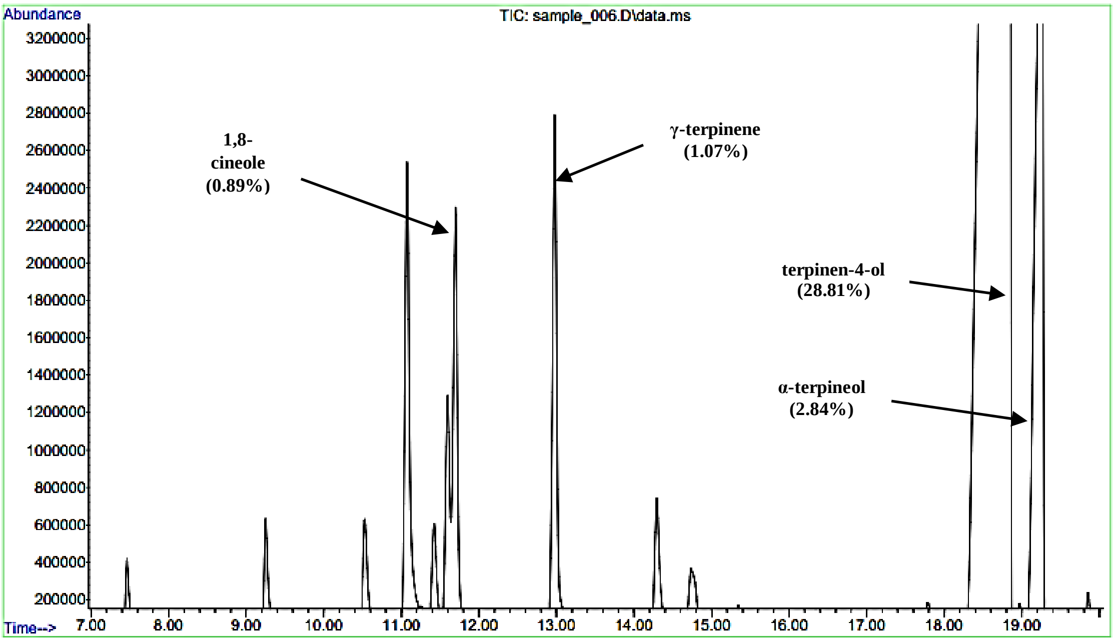

FIGURE 4.3 Chromatographic profile of tea tree absolutes from petroleum ether extraction

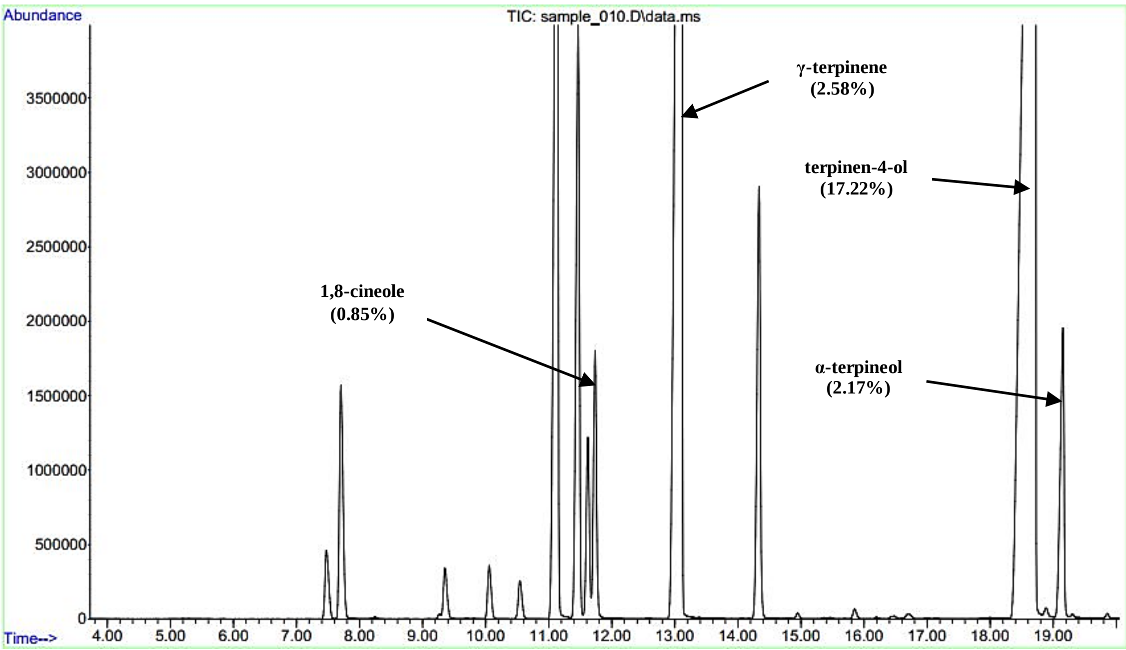

FIGURE 4.4 Chromatographic profile of tea tree absolutes from ethanol extraction

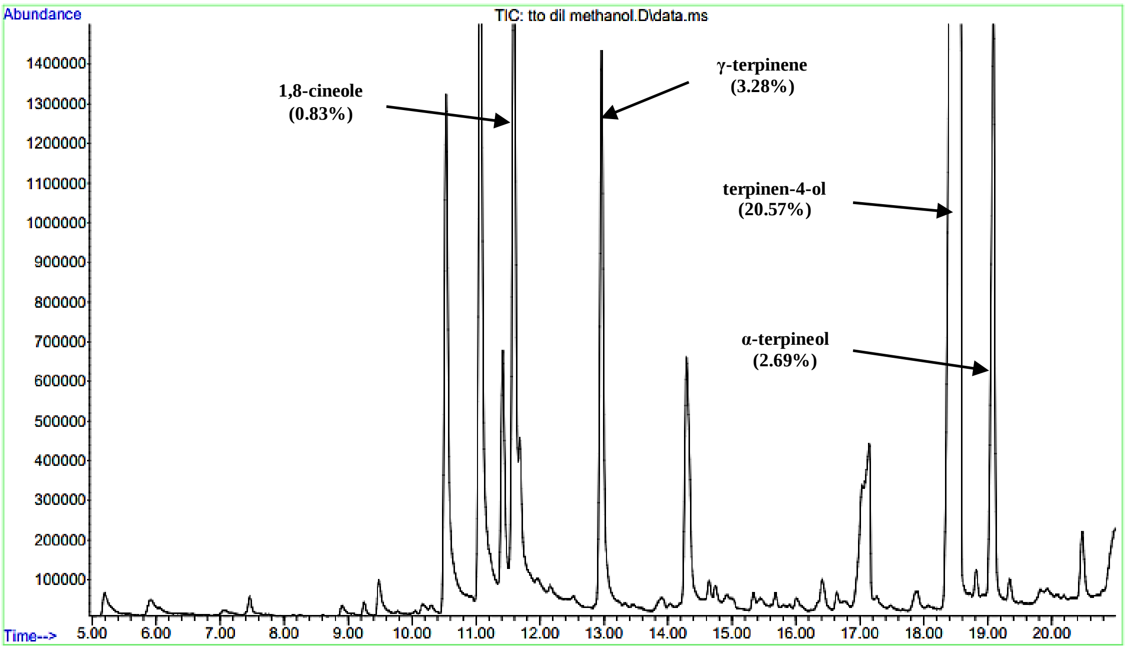

FIGURE 4.5 Chromatographic profile of tea tree absolutes from methanol extraction

TABLE 4.1 Yield of absolutes from Melaleuca alternifolia sp. based on different types of solvents

Extraction Solvents	Mass of absolute extracted (g/g)	Yield of absolute on a fresh weight basis (%)
n-Hexane	0.48	0.96
Petroleum Ether	0.53	1.06
Ethanol	0.39	0.78
Methanol	0.39	0.78

TABLE 4.2 Monoterpenes composition from GC-MS characterization

Monoterpenes	Standard TTO (%)	Extracted TTO (%)				ISO 4730 Standard Range (%)
		n-hexane	petroleum ether	ethanol	methanol	
terpinen-4-ol	39.44	39.34	28.81	17.22	20.57	30-48
1,8-cineole	2.22	1.46	0.89	0.85	0.83	trace-15
α-terpineol	2.90	3.70	2.84	2.17	2.69	1.5-8
γ-terpinene	20.47	1.49	1.07	2.58	3.28	10-28

In this research project, the solvent extraction method were carried out based on two classes of solvent, which includes both non-polar solvents (n-hexane and petroleum ether) and polar solvents (ethanol and methanol). Among these solvents, it was claimed that petroleum ether provides the highest yield of tea tree oil in comparison to the yield extracted based from n-hexane, ethanol and methanol solvents. Table 4.1 shows the data tabulations obtained on the yield of tea tree absolutes from *Melaleuca alternifolia sp.* based on the different types of solvents studied, which are expressed in terms of mass of absolutes extracted (g) as well as in yield percentage (%) on a basis of fresh weight of initial sample. As stated by (Danlami, Arsad, & Zaini, 2015), the percentage yield of the extracted absolute from the plant sample can be calculated by using the following formula:

$$\text{Yield of extraction (\%)} = \frac{\text{Weight of extracted oil}}{\text{Weight of total sample}} \times 100\% \quad (1)$$

For instance, as the *Melaleuca alternifolia sp.* sample is extracted by using petroleum ether solvent, the yield of absolute being extracted is calculated as shown in the calculation below:

$$\text{Yield of extraction} = \frac{0.53\text{g}}{50\text{g}} \times 100\% \qquad (2)$$
$$= 1.06\%$$

Petroleum ether extraction provides a yield of 0.53 g of oil / g of tea tree sample, which was 10.42%, significantly higher than the amount of oil extracted in n-hexane extraction. Similarly, the yield of tea tree absolutes from petroleum ether extraction also showed about 35.90%, significant increment in terms of comparing its corresponding yield with respect to the yield of absolutes from both ethanol and methanol extraction (Baker, Lowe, & Southwell, 2000). Hence, it could be said that petroleum ether solvent is a better choice of solvent, followed by n-hexane solvent extraction as compared to both ethanol and methanol extraction of tea tree absolutes. This observation can be further explained based by the greater interaction of monoterpene hydrocarbons towards non-polar solvents (like dissolve like), instead of solubilizing in polar solvents such as ethanol and methanol (Carvalho, Galvao, Barros, Conceicao, & Sousa, 2012).

As for the characterization study purposes, a total of four monoterpene compounds were identified from the extracted tea tree absolutes based on different types of solvents, together with their corresponding percentage composition, were shown in Table 4.2. The monoterpenes identified were claimed to contribute towards the antimicrobial strength of the tea tree absolutes in which, terpinen-4-ol and 1,8-cineole monoterpenes were the major components whereas α-terpineol and γ-terpinene were to be the minor components. A standard tea tree oil, purchased from Biossentials Sdn Bhd, was used as the "benchmark" study to compare the relative compositions of the monoterpenes compounds with respect to the composition obtained in the extracted tea tree oil. From Table 4.2, the major component, terpinen-4-ol, from n-hexane extraction showed the highest composition and almost close to the standard tea tree oil (TTO) composition as compared to the composition found in petroleum ether, ethanol and methanol. Similarly, the composition of both 1,8-cineole and α-terpineol were found to be the highest and also in agreement with the standard TTO composition as well as in accordance to the ISO 4730 *Melaleuca* oil standard.

In the case of γ-terpinene compound, the overall content identified from the extraction of the studied solvents were proven to be at a very minimum amount (1.49 - 3.28%) as compared to the standard TTO composition (20.47%) and the γ-terpinene composition range regulated in ISO 4730 oil standard (10-28%). This could be possibly due to the circumstances in which during the extraction process, the composition of the extracted tea tree oil changes in the presence of oxygen and also other environmental factors such as being exposed to light. As such, the levels of α-terpinene, γ-terpinene and terpinolene decreases whereas the level of p-cymene and terpinen-4-ol increases up to tenfold. Subsequently, the hydrolytic conversion and oxidative degradation leads to the formation of peroxides, endoperoxides and epoxides which in turn reduces the content of γ-terpinene, thus converting this reduction and contributing to the surplus composition of terpinen-4-ol and p-cymene as shown in Figure 4.6 below (Chambers, et al., 2004) (Southwell, Leach, Lowe, & Pollack, 2006):

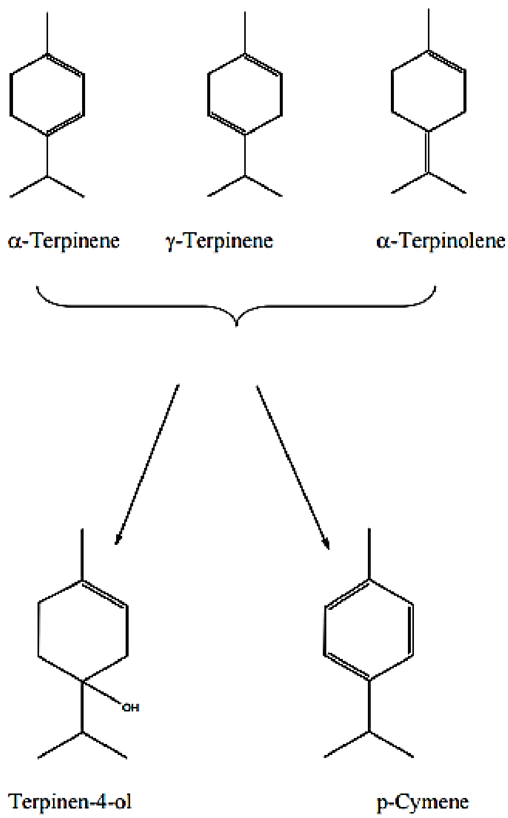

FIGURE 4.6 End products of hydrolysis and oxidation of tea tree oil constituents

CHAPTER 5

CONCLUSION

The extraction of essential compounds from herbaceous plants, such as *Melaleuca alternifolia*, contains thousands of valuable constituents of naturally active molecules displaying their antimicrobial properties which could be harnessed to treat various illnesses and infections. As such, the essential oil extracted from *Melaleuca alternifolia* genus containing these antimicrobial properties are studied in greater details and established to be the precursor for the development of various antidotes and/or antibiotics in the pharmaceutical industries. In the present research work, the yield of the absolutes is significantly higher by using non-polar solvents as compared to polar solvent, in which petroleum ether was claimed to be the best solvent for the extraction purposes. However, in terms of the composition analysis, the composition of the monoterpenes compounds (terpinen-4-ol, 1,8-cineole and α-terpineol) were in accordance to the standard tea tree oil as well as with the composition range being regulated under the ISO 4730 *Melaleuca* oil standard. However, additional consideration or project work is required in the upcoming research work to further investigate the degradation effect of γ-terpinene compound in tea tree oil, which could possibly result in losses of valuable antimicrobial properties of the therapeutic oil. For future recommendation purposes, instead of using non-polar solvents as the extraction medium, ionic liquid as well as green solvents such as d-limonene, can also be used, to evaluate their efficiency as an extraction medium in providing the yield of essential oil of *Melaleuca alternifolia sp.* as they are less harmful to human health, lower toxicity level and more eco-friendly as compared to non-polar solvents (Ali & Inamuddin, 2012).

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